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Two glycerol uptake systems contribute to the high osmotolerance of *Zygosaccharomyces rouxii*

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Summary

The accumulation of glycerol is essential for yeast viability upon hyperosmotic stress. Here we show that the osmotolerant yeast *Zygosaccharomyces rouxii* has two genes, *ZrSTL1* and *ZrSTL2*, encoding transporters mediating the active uptake of glycerol in symport with protons, contributing to cell osmotolerance and intracellular pH homeostasis. The growth of mutants lacking one or both transporters is affected depending on the growth medium, carbon source, strain auxotrophies, osmotic conditions and the presence of external glycerol. These transporters are localised in the plasma membrane, they transport glycerol with similar kinetic parameters and besides their expected involvement in the cell survival of hyperosmotic stress, they surprisingly both contribute to an efficient survival of hypoosmotic shock and to the maintenance of intracellular pH homeostasis under non-stressed conditions. Unlike *STL1* in *Sa. cerevisiae*, the two *Z. rouxii STL* genes are not repressed by glucose, but their expression and activity are downregulated by fructose and upregulated by non-fermentable carbon sources, with *ZrSTL1* being more influenced than *ZrSTL2*. In summary, both transporters are highly important, though *Z. rouxii* CBS 732[T] cells do not use external glycerol as a source of carbon.

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Introduction

Tolerance to changes in environmental water activity is one of the key factors enabling microorganisms, including yeasts, to survive in nature. Water activity is the chemical potential of free water in solution. As the range of water activity may change very rapidly (rain, desiccation, etc.), microorganisms have developed sophisticated strategies to adapt and survive to these changes. Upon hypoosmotic stress, i.e. high water activity, water penetrates into the cytosol following its concentration gradient and cells have a tendency to swell. During hyperosmotic stress (low external water activity), water leaves the cells and they shrink. To prevent excessive changes in the intracellular water concentration, cells correlate the concentration of compatible solutes in the cytosol with the external water activity. The strategy for accumulating compatible solutes in osmoadaptation is relatively conserved in bacteria, archea and eukaryota, although the solutes differ (Yancey *et al.*, 1982; Yancey, 2005; Saito and Posas, 2012). Glycerol, as a small and uncharged molecule, is the main and most frequently used compatible solute in yeast species (Brown, 1978; Edgley and Brown, 1978; Neves *et al.*, 2004). On the one hand, external glycerol may serve as a source of carbon for growth; and on the other, yeasts produce it for osmoadaptation purposes, for maintaining the redox balance (Vandijken and Scheffers, 1986; Ansell *et al.*, 1997; Larsson *et al.*, 1998), in response to temperature (Izawa *et al.*, 2004a,b) and oxidative stresses (Pahlman *et al.*, 2001), and last, but not least as a precursor of glycerolphospholipid synthesis (Hohmann, 2002; Czabany *et al.*, 2007). The ability to both produce and consume glycerol, together with its broad-scale use in cell metabolism and physiology requires a tight regulation of its synthesis, catabolism, uptake and release. Among the key factors, regulating the glycerol content in yeast cells is the mitogen-activated protein kinase HOG signalling pathway (Brewster and Gustin, 2014) and two types of transporters in the yeast plasma membrane, an active importer [Stl1; (Ferreira *et al.*, 2005)] and an efflux channel [Fps1; (Tamas *et al.*, 1999)]. Upon hyperosmotic shock, the HOG pathway is activated and it helps the cells: (i) to immediately close the transporters through which the compatible solutes [glycerol via Fps1p; (Ahmadpour *et al.*, 2014) or

ions [potassium via Nha1p and Tok1p; (Proft and Struhl, 2004; Kinclova-Zimmermannova and Sychrova, 2006)] may be lost, (ii) to upregulate the synthesis of a glycerol-importing protein [Stl1; (Rep *et al*., 2000; Ferreira *et al*., 2005)] and (iii) to increase the synthesis of glycerol (Hohmann, 2002).

Osmotolerant yeast species produce, in general, less glycerol upon osmotic stress, but are able to accumulate and retain it in their cytosol more effectively than the conventional yeasts *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe* (Edgley and Brown, 1978; van Zyl and Prior, 1990). The true nature of intrinsic osmotolerance is not well understood as some salt-tolerant yeast species, living at the boundaries of salt solubility, maintain similar intracellular glycerol homeostasis to *Sa. cerevisiae* (Silva-Garca and Lucas, 2003; Silva-Graca *et al*., 2003). An effective active reuptake of synthesised and lost glycerol is believed to be one of the factors enabling some yeast species to survive with a relatively low energy cost for glycerol synthesis in an environment with a high osmotic pressure. This hypothesis was confirmed by a large study measuring the active uptake and accumulation of glycerol in more than 40 yeast species (Lages *et al*., 1999) showing the existence of efficient transport systems mediating the uptake of glycerol in symport with sodium cations or protons in osmotolerant yeast species.

The existence of an active glycerol uptake system in *Sa. cerevisiae* had been known from kinetic studies (Lages and Lucas, 1997; Lages *et al*., 1999) for many years, but it was only 10 years ago that the gene and corresponding protein were identified and their function confirmed experimentally (Ferreira *et al*., 2005). *Sc*Stl1p is a glucose-regulated glycerol/H^+ symporter whose structure resembles the structure of hexose transporters (Nelissen *et al*., 1997). In the presence of glucose, the *ScSTL1* gene is repressed and the already existing molecules of the transporter are inactivated and after internalisation degraded in vacuoles (Ferreira *et al*., 2005). On the other hand, the expression of *ScSTL1* is strongly induced by non-fermentable carbon sources and upon entry to the stationary phase of growth (Lages and Lucas, 1997; Rep *et al*., 2000; Ferreira *et al*., 2005; Roberts and Hudson, 2006). Moreover, hyperosmotic conditions and high temperature induce the synthesis of this symporter as well (Rep *et al*., 2000; Ferreira and Lucas, 2007).

Although homologues of *ScSTL1* gene apparently exist in the genomes of almost all yeast species, Stl1, proteins have only been fully characterised in *Sa. cerevisiae* (Ferreira *et al*., 2005) and *Candida albicans* (Kayingo *et al*., 2009). In *C. albicans*, two *STL* genes were identified and cloned, and their products were characterised together with corresponding deletion mutants. Unlike *ScSTL1*, *CaSTL1* and its product are not affected by glucose. The second *C. albicans* gene, *CaSTL2*, does not encode a glycerol transporter, although there is some evidence that it is involved in glycerol homeostasis (Kayingo *et al*., 2009). The presence of active glycerol uptake systems was, based on kinetics measurements, also confirmed in other yeast species (Lages *et al*., 1999) and biochemically characterised in *Debaryomyces hansenii* (Lucas *et al*., 1990), *Pichia sorbitophila* (Lages and Lucas, 1994; 1995) and *Zygosaccharomyces rouxii* (van Zyl *et al*., 1990). On the other hand, an active glycerol uptake was neither observed in the fission yeast *Sc. pombe* (Lages *et al*., 1999; Palma *et al*., 2007) nor were *STL*-like coding sequences identified in the genomes of *Sc. pombe* and the opportunistic pathogen *Candida glabrata* (Palma *et al*., 2007). A detailed characterisation of glycerol transporters in non-conventional yeasts is hampered due to the lack of tools for genetic manipulation (mainly gene deletion). Thus, the only possibility is heterologous expression in *Sa. cerevisae*, which was used e.g. for *STL* genes from *D. hansenii* and *Pachysolen tannophilus* (Liu *et al*., 2013; Pereira *et al*., 2014).

Zygosaccharomyces rouxii is a hemiascomycetous yeast closely related to the model yeast *Sa. cerevisiae*. In contrast to glucophilic *Sa. cerevisiae*, *Z. rouxii* consumes fructose faster than glucose, i.e. it is fructophilic (Emmerich and Radler, 1983; Leandro *et al*., 2014). It is notoriously known for its ability to spoil many food products with high concentrations of sugar such as fruit juices, syrups, dressings, ketchup and sweet sauces. The unusual physiological characteristics of this yeast, including resistance to weak acids, high temperature and an extreme osmotolerance are largely responsible for its ability to cause this spoilage (Fleet, 1992; Martorell *et al*., 2007). On the other hand, the same physiological characteristics underlie its broad use in the food industry, e.g. in the production of soy sauce, Miso paste or balsamic vinegar (Onishi, 1963; Solieri *et al*., 2006; Dakal *et al*., 2014). Its extreme osmotolerance allows *Z. rouxii* to grow in an environment with high concentrations of sugar and/or salts, which normally restricts the growth of most other yeast species.

As for glycerol metabolism and transport in *Z. rouxii*, as in *Sa. cerevisiae*, *Z. rouxii* employs two different mechanisms for glycerol transport and possesses the members of the HOG pathway (Iwaki *et al*., 1999). For glycerol efflux, *Zr*Fps1p is a low-affinity transporter releasing glycerol from cells by facilitated diffusion. It conserves the structural features and regulatory mechanisms of *Sc*Fps1p, i.e. hyperosmotic conditions cause closing, and hypoosmotic stress induces opening of this channel (Tang *et al*., 2005). The active uptake of glycerol was studied intensively in this species, and its accumulation in cells was believed to be driven by a symport with Na^+ (van Zyl *et al*., 1990; Lages *et al*., 1999). Despite all similarities with *Sa. cerevisiae*, there are several pieces of strong evidence for a different

Table 1. List of strains.

Strain	Genotype	Source/reference
Zygosaccharomyces rouxii		
CBS 732^T	Wild type	CBS collection
UL4	CBS 732^T *ura3*	Pribylova and Sychrova (2003)
MS1	UL4 *stl1Δ::loxP*	This study
MS2	UL4 *stl2Δ::loxP*	This study
MS12	UL4 *stl2Δ::loxP stl1Δ::kanMX*	This study
DLA2	*ura3 leu2Δ::loxP ade2Δ::loxP*	Pribylova *et al.* (2007b)
DLAM1	DLA2 *stl1Δ::loxP*	This study
DLAM2	DLA2 *stl2Δ::loxP*	This study
DLAM12	DLA2 *stl1Δ::loxP stl2Δ::kanMX*	This study
Saccharomyces cerevisiae		
fps1Δ	W303-1A *fps1Δ::LEU2*	M. Duskova, unpubl. obs.
fps1Δ stl1Δ	W303-1A *fps1Δ::LEU2 stl1Δ::kanMX*	M. Duskova, unpubl. obs.
fps1Δ stl1Δ::ZrSTL1	W303-1A *fps1Δ::LEU2 stl1Δ::ZrSTL1-TPS1^T-kanMX*	M. Duskova, unpubl. obs.
fps1Δ stl1Δ::ZrSTL2	W303-1A *fps1Δ::LEU2 stl1Δ::ZrSTL2-TPS1^T-kanMX*	M. Duskova, unpubl. obs.
fps1Δ stl1Δ::ScSTL1	W303-1A *fps1Δ::LEU2 stl1Δ::ScSTL1-TPS1^T-kanMX*	M. Duskova, unpubl. obs.
hog1Δ stl1Δ	W303-1A *hog1Δ::LEU2 stl1Δ::kanMX*	M. Duskova, unpubl. obs.
BW31a	W303-1A *nha1::LEU2 ena1Δ::HIS3::ena4Δ*	Kinclova-Zimmermannova *et al.* (2005)
BY4741	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF collection
stl1Δ	BY4741 *stl1Δ::loxP*	Duskova *et al.* (2015)
AQ	*ura3*	A. Querol, Valencia

^{T}, terminator.

regulation of glycerol metabolism and transport in *Z. rouxii* (van Zyl *et al.*, 1991; Iwaki *et al.*, 2001; Pribylova *et al.*, 2007a).

Taking advantage of the sequenced genome (Sherman *et al.*, 2009) and the existence of tools for the genetic manipulation of *Z. rouxii* (Pribylova and Sychrova, 2003; Pribylova *et al.*, 2007b,c), we characterised two *Z. rouxii* glycerol-importing systems (*Zr*Stl1 and *Zr*Stl2) in this study. To characterise their transport activity and physiological function, we constructed single- and double-deletion mutants of *Z. rouxii*, described their growth and stress-tolerance phenotypes, measured the uptake of glycerol and estimated their expression under various conditions. To compare the Stl proteins' transport activity, we also expressed the two *Z. rouxii* genes in *Sa. cerevisiae* cells. Our results showed that both genes encode functional glycerol uptake systems, which differ in their physiological role and regulation of expression. Taken together, *Z. rouxii* Stl transporters fulfil a very important role in the physiology of this osmotolerant yeast species, and contribute to its 'spoiling' nature.

Results

Z. rouxii *possesses two highly similar homologues of the* ScSTL1 *gene*

Although the existence of an efficient active uptake of glycerol in *Z. rouxii* as well as in some other osmotolerant yeast species was suggested in the past (van Zyl *et al.*, 1990; Lages and Lucas, 1995; Lages *et al.*, 1999), only two systems have been characterised so far [Stl1 transporters from *Sa. cerevisiae* and *C. albicans*; (Ferreira *et al.*, 2005; Kayingo *et al.*, 2009)]. We found two open-reading frames similar to the *ScSTL1* and *CaSTL1* genes in the genome of *Z. rouxii* CBS 732^T. The ZYRO0E01210g ORF (Gene ID: 8204531) and the ZYRO0E01188g ORF (Gene ID: 8204530) are localised in tandem on the Zyro0E chromosome and we named them *ZrSTL1* and *ZrSTL2*, respectively. At the protein level, both *Z. rouxii* Stl proteins share 67% identity (70% if sequences without the hydrophilic termini are compared), and their identity with *Sc*Stl1p and *Ca*Stl1p ranges from 57% to 63%. They probably contain 12 transmembrane segments as well as five motifs conserved in the family of yeast sugar transporters [Fig. S1 and Leandro *et al.* (2009)]. It is worth noting that the hydrophilic N-terminus of *Zr*Stl2p is 87 amino acids longer than that of *Zr*Stl1p (Fig. S1).

Only Zr*Stl1p is fully functional in* Sa. cerevisiae *and transports glycerol similarly to* Sc*Stl1p*

To characterise and compare the properties of the products of *ZrSTL1* and *ZrSTL2* genes, we first used heterologous expression in *Sa. cerevisiae*. The *Sa. cerevisiae* W303-derived strains used lacked their own *STL1*, *FPS1* and *HOG1* genes in various combinations (Table 1), and thus were unable to import and/or export glycerol, increase its production upon hyperosmotic stress and consequently were more osmosensitive than the parental strain. Both *ZrSTL* genes were either cloned in multicopy YEp352-based plasmids (Table S1) behind a weak and constitutive

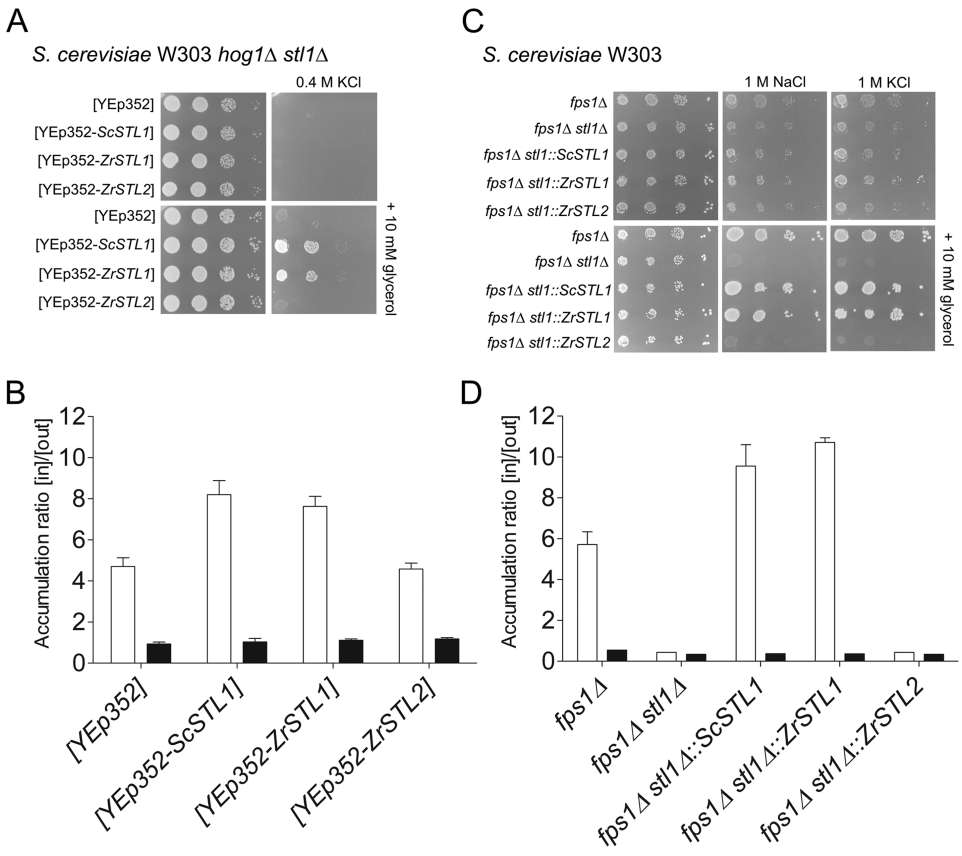

Fig. 1. Expression of *ZrSTL1* and *ZrSTL2* in *Saccharomyces cerevisiae*.
A. Growth phenotypes of *Sa. cerevisiae* W303 *hog1Δ stl1Δ* transformed with empty vector YEp352 or its derivatives with the three *STL* genes YEp352-*ScSTL1*, YEp352-*ZrSTL1*, YEp352-*ZrSTL2*. Cells were grown on solid YNB media (2% mannitol, BSM without uracil) with KCl and glycerol as indicated.
B. Accumulation ratios of ^{14}C-glycerol in *Sa. cerevisiae* W303 *hog1Δ stl1Δ* (transformed with plasmids as in A) grown in YEPG (open bars) or YNB with 2% glucose (black bars). The plot shows the accumulation ratios after 45 min. Each bar represents a mean ± SD of three biological replicates.
C. Growth phenotypes of *Sa. cerevisiae* W303 derivatives *fps1Δ*, *fps1Δ stl1Δ*, *fps1Δ stl1::ScSTL1*, *fps1Δ stl1::ZrSTL1* and *fps1Δ stl1::ZrSTL2*. Cells were grown on solid YNB media (2% mannitol, BSM) supplemented with salts and glycerol as indicated.
D. Accumulation ratios of ^{14}C-glycerol in *Sa. cerevisiae* W303 derivatives *fps1Δ*, *fps1Δ stl1Δ*, *fps1Δ stl1::ScSTL1*, *fps1Δ stl1::ZrSTL1* and *fps1Δ stl1::ZrSTL2* grown in YEPG (open bars) or YPD (black bars). The plot shows the accumulation ratios after 45 min. Each bar represents a mean ± SD of three biological replicates.

ScNHA1 promoter (Banuelos *et al.*, 1998) or integrated into the *ScSTL1* locus. As positive controls, similar episomal and integrative constructs with *ScSTL1* were used (Table 1 and Table S1). The transformation of strains with the empty YEp352 and a *Sa. cerevisiae* mutant lacking its own *STL1* gene served as negative controls, respectively. The ability of the constructed strains expressing *ZrSTL* genes to grow under conditions differing in their available carbon source (fermentative or respirative) and solutes (KCl, NaCl, sorbitol) used for osmotic stress was determined in a series of drop tests. The results obtained with cells transformed with multicopy plasmids showed that only the expression of *Zr*Stl1 or *Sc*Stl1 complemented the high osmosensitivity of a *Sa. cerevisiae hog1Δ stl1Δ* strain, and enabled it to grow in the presence of salts. The full complementation occurred if the growth medium was supplemented with a low concentration of glycerol, as shown in Fig. 1A for cells growing on 2% mannitol and 0.4 M KCl. Similar results for cells expressing *ZrSTL1* or *ScSTL1*, i.e. improved growth in the presence of low concentrations of glycerol, were obtained when media with glucose and NaCl were used (not shown). The expression of *ZrSTL2* from a multicopy plasmid did not show a clear phenotype under any of the tested conditions. To confirm the ability of *Zr*Stl1p to transport glycerol, we first measured the accumulation of radiolabelled glycerol in cells harbouring the *STL* genes on multicopy plasmids and grown either with fermentative or respirative carbon sources. As shown in Fig. 1B, the accumulation of glycerol was significantly higher in cells expressing *Zr*Stl1p and *Sc*Stl1p than in cells transformed

with an empty plasmid or with a plasmid harbouring the *ZrSTL2* gene. This difference was only observed if the cells were grown in a media with a non-fermentable source of carbon (Fig. 1B, white bars). When the cells were grown in the presence of glucose, no accumulation was observed (Fig. 1B, black bars) which suggested that *Zr*Stl1p might be inactivated by glucose, like *Sc*Stl1p. Similar results were obtained when cells with *Z. rouxii* genes integrated into the *ScSTL1* locus were compared (Fig. 1C and D). The presence of both *STL1* genes (but not that of *ZrSTL2*) improved, in the presence of a low amount of added glycerol, the tolerance of cells to salts and these cells accumulated a much higher amount of external glycerol. Surprisingly, the reintegration of *ScSTL1* to its original locus resulted in cells with a higher accumulation of glycerol than that observed for cells with the original *ScSTL1* locus (cf. Fig. 1D *fps1Δ* vs. *fps1Δ stl1::ScSTL1*). This result was most likely due to the use of the *ScTPS1* terminator (Yamanishi *et al*., 2011) in the integrative plasmid with *ScSTL1* (M. Duskova, unpublished).

Altogether, the expression of *Z. rouxii STL* genes in *Sa. cerevisiae* revealed that *Zr*Stl1p is a glycerol transporter with similar properties (putative inactivation by glucose in *Sa. cerevisiae* cells) to *Sc*Stl1p, and that *Zr*Stl2p is either not properly expressed in *Sa. cerevisiae* or does not function as a glycerol transporter. Green Fluorescent Protein (GFP) tagging of *Zr*Stl1 and *Zr*Stl2 coding sequences revealed that ZrStl1 is properly localised in *the Sa. cerevisiae* plasma membrane, whereas no fluorescence signal was observed in cells transformed with a plasmid encoding *Zr*Stl2-GFP protein (not shown).

Z. rouxii *mutants lacking* STL *genes are more sensitive to changes in external osmolarity, produce more and accumulate less glycerol*

To construct *Z. rouxii* mutants lacking *STL* genes we used the UL4 (*ura3*) and DLA2 (*ade2 leu2 ura3*) strains, derived from CBS 732^T (Table 1). When we compared the growth of the three strains and their salt tolerance, it was evident that the auxotrophic mutations were not innocuous. They produced a slower growth under standard conditions [YNB supplemented with 2% glucose and Brent Supplement Mix (BSM)] and an increased sensitivity to salt stress. The multiple auxotrophies had a more pronounced effect than the single *ura3* mutation (Fig. S2A). We constructed single- and double-deletion mutants, lacking *STL1* and/or *STL2* genes, in both the UL4 and DLA2 backgrounds. When the growth of the resulting *Z. rouxii* single (*stl1Δ* or *stl2Δ*) and double (*stl1Δ stl2Δ*) mutants was compared under standard non-stress conditions (YNB supplemented with 2% glucose and BSM), it was evident that the deletion of *STL* genes slightly inhibited the growth of UL4-derived mutants (Fig. 2A, left panel, last drops), and the deletion of *STL1* severely affected the growth of DLA2-derived mutants (Fig. S2B, right panel). This phenotype was visible on YNB medium and not on rich YPD (Fig. S2B, left panel). These results suggested that the *Z. rouxii* Stl transporters, mainly Stl1p, are not only necessary for growth under salt stress as predicted, but also in its absence, under standard conditions, which suggests glycerol might be important for *Z. rouxii* fitness on YNB with glucose. When we compared the osmotolerance of the mutants on plates containing higher concentrations of salts, it was evident, on both YNB and YPD media, that it was mainly the lack of *STL1* that rendered the *Z. rouxii* cells more sensitive to salts, and that the deletion of both *STL* genes resulted in a low salt tolerance. An example of the results obtained with DLA2-derived strains growing on YPD medium in the presence of 2.5 M KCl is shown in Fig. S2B, middle panel; similar results were obtained with NaCl or sorbitol (not shown). As the DLA2 growth was compromised under almost all conditions tested, only UL4 and its mutants were used for the rest of experiments.

The assumption of Stl function under non-stressed conditions was confirmed in an experiment where we estimated the amount of glycerol produced by UL4 and derived mutants growing under non-stressed conditions (YPD medium; $A_{600} = 1$). Figure 2B shows that the MS1 and MS12 mutants (lacking *STL1*) produced significantly more glycerol than UL4 and MS2. We also measured the accumulation of glycerol in UL4 and derived mutants and we obtained several interesting results. As we supposed, based on our experiments with the expression of *ZrSTL* genes in *Sa. cerevisiae*, the deletion of *ZrSTL1* resulted in a diminished ability to accumulate external glycerol (Fig. 2C, UL4 vs. MS1). However, the mutant with deleted *ZrSTL2* also accumulated significantly less glycerol than the parental strain (Fig. 2C UL4 vs. MS2), and the double MS12 mutant was unable to accumulate glycerol inside its cells. This was the first indication that *Zr*Stl2p is also a glycerol transporter. It is worth noting that these results obtained with cells grown in YPD suggested that, unlike in *Sa. cerevisiae*, the Stl transporters were neither repressed nor inactivated by glucose in *Z. rouxii* cells.

When testing the tolerance of strains lacking the *STL* genes to a hyperosmotic stress, we also tested their ability to survive hypoosmotic shock. Testing the ability to survive a long hyperosmotic [YP + 1 M (18%) mannitol, 16 h] stress followed by a short hypoosmotic shock (H_2O, 45 min), we saw a surprising phenotype. Deletion of the *STL* genes did not change the ability of cells to survive a mild hyperosmotic stress, but it significantly affected the number of cells surviving the hypoosmotic shock (Fig. 2D). The double mutant had the most difficulty surviving a hypoosmotic stress and this phenotype was cumulative in terms of the survival of single and double mutants, respectively (Fig. 2D). Similar results were obtained when

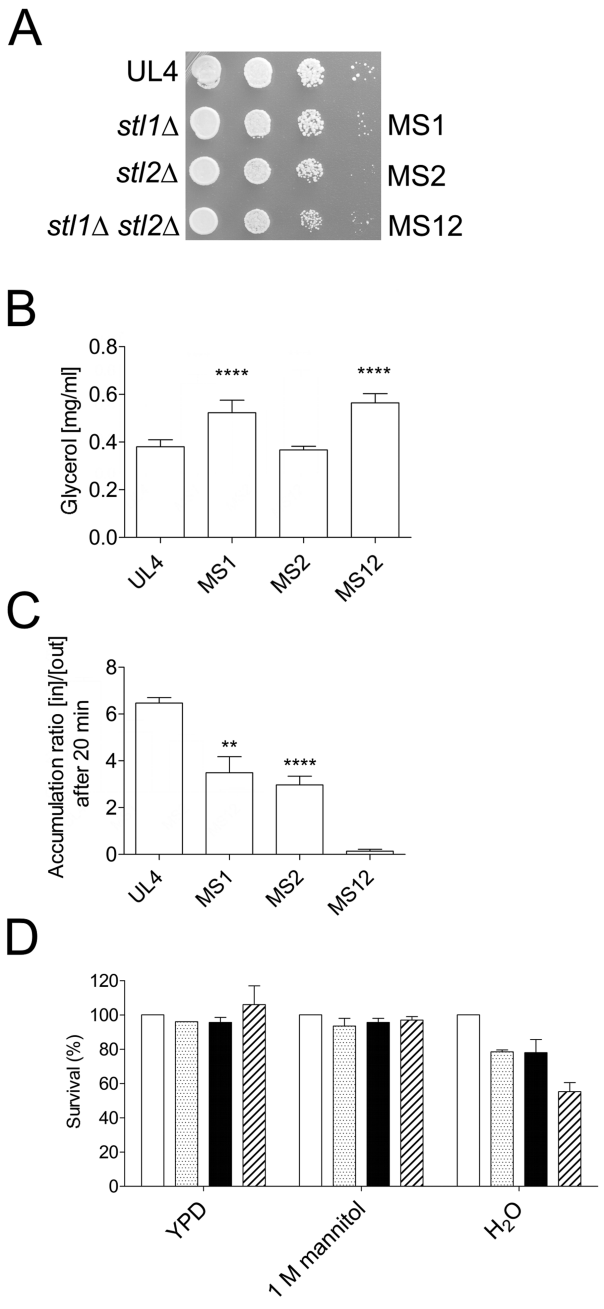

Fig. 2. Growth phenotypes, accumulation and production of glycerol, and stress tolerance of *Zygosaccharomyces rouxii* UL4 and *stl* mutants (MS1, *stl1Δ*; MS2, *stl2Δ*; MS12, *stl1Δ stl2Δ*).
A. Growth on solid YNB media supplemented with 2% glucose and BSM.
B. Total concentration of glycerol produced by cell cultures grown up to $A_{600} = 1$ in YPD. The error bars represent the means of the results of three biological replicates assayed in triplicate ± SD. ****, $P < 0.0001$ (two-tailed Student's *t*-test versus *Z. rouxii* UL4).
C. Accumulation ratios of ^{14}C-glycerol in cells grown in YPD. The bars represent the accumulation ratio after 20 min. Presented results are the means ± SD of three biological replicates. **, $P < 0.01$; **** $P < 0.0001$ (two-tailed Student's *t*-test versus UL4).
D. Survival of combination of hyperosmotic (16 h in YP + 1 M mannitol) and subsequent hypoosmotic (45 min in distilled water) conditions evaluated in a CFU assay. The CFU was estimated after each step of the experiment. CFU of UL4 (open bars) represent 100% of survivors. MS1, *stl1Δ*, shaded bars; MS2, *stl2Δ*, black bars; MS12, *stl1Δ stl2Δ*, hatched bars.

YPD + 1M KCl instead of YP + 1 M mannitol was used (data not shown). In summary, both Stl transporters contributed to the ability to survive hypoosmotic stress.

Z. rouxii *strains lacking* STL *genes are highly osmosensitive*

A high accumulation of glycerol was usually observed for cells incubated in the presence of high concentrations of NaCl (van Zyl *et al.*, 1990; Lages *et al.*, 1999). Thus, considering the Stl proteins as glycerol uptake systems, deletion of the *STL* genes should mainly affect the growth of *Z. rouxii* cells in the presence of salts. This was tested in a series of drop tests on plates supplemented with various solutes to elucidate whether the mutant phenotype was salt-specific. Besides relatively toxic NaCl, high concentrations of non-toxic KCl or of a non-salt solute (sorbitol or mannitol) were tested. The results from our previous work had shown that a slightly higher osmolarity (approx. 0.3–0.5 M of NaCl) supported the growth of *Z. rouxii* CBS 732^T on YNB medium (Bubnova *et al.*, 2014). Here we observed a similar phenotype of improved growth on YNB plates supplemented with 0.6 M KCl, and not only for the UL4 parental strain, but surprisingly for the three mutants as well (Fig. 3B, left and middle panels). A higher osmotic pressure, e.g. 1.5 M sorbitol (but also 1 M KCl, not shown), only significantly affected the growth of the MS12 double mutant (Fig. 3A and B, right panels). These results suggested that the ability of *Z. rouxii* to grow better in the presence of mild salt concentrations does not depend on the presence of *STL* genes, and simultaneously, that one *STL* gene is enough to ensure *Z. rouxii* growth under a strong hyperosmotic stress caused by non-charged solutes like sorbitol.

We also tested the influence of various solutes (simultaneously carbon sources and osmotic stress inducers, e.g. mannitol, sorbitol, maltose, sucrose or galactose) on the osmotolerance of UL4 and the derived mutants (typical results are shown in Fig. 3C). When high concentrations of sugars or polyols were used (e.g. Fig. 3C, 1 M mannitol or 1 M galactose), the growth of all tested strains was inhibited when no glycerol was added to the medium. Upon the addition of only 10 mM glycerol, the UL4 strains and the two mutants possessing one of the two Stl proteins were able to grow. The growth of the double mutant lacking both *STL* genes was not restored by the presence of glycerol (Fig. 3C, two panels on the right). This result confirmed the role of both Stl proteins in the general osmotolerance (and not only the salt tolerance) of

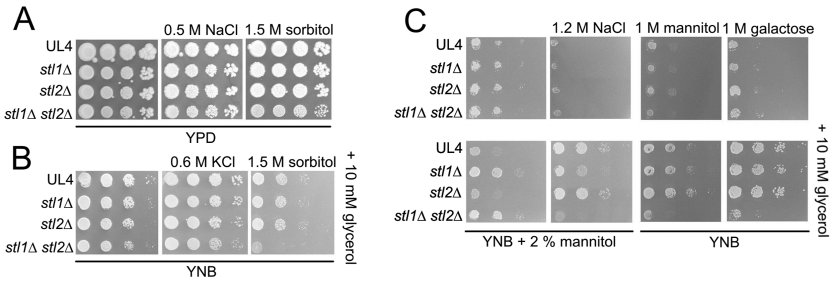

Fig. 3. Osmotolerance of *Zygosaccharomyces rouxii* UL4 and its *stl* derivatives (MS1, *stl1Δ*; MS2, *stl2Δ*; MS12, *stl1Δ stl2Δ*).
A. Growth phenotypes on solid YPD media supplemented with salt or sorbitol as indicated.
B. Growth phenotypes on YNB supplemented with 2% glucose and salt or sorbitol as indicated.
C. Effect of glycerol addition on osmotic stress tolerance. Growth phenotypes on YNB supplemented with mannitol or galactose as carbon sources, salt and glycerol as indicated.

Z. rouxii cells. When 2% mannitol was used as a carbon source, both expected and surprising results were obtained (Fig. 3C, two left panels). As we expected, the presence of the *ZrSTL1* gene (UL4 and MS2 strains) significantly improved the growth phenotypes in the presence of a high salt concentration and 10 mM glycerol. This result indicated that *Zr*Stl1 probably plays a more important role in glycerol uptake than the *ZrSTL2* gene when cells use mannitol as a source of carbon. Simultaneously, a very interesting and unexpected phenotype was observed on YNB medium with 2% mannitol as a source of carbon and 10 mM glycerol, i.e. with no hyperosmotic stress (Fig. 3C, left bottom panel). Cells lacking *ZrSTL1* (MS1 and MS12) grew better than cells with a functional Stl1 glycerol transporter. This phenotype suggested that the uptake of glycerol via Stl1p when *Z. rouxii* cells grew on mannitol and without an osmotic stress was disadvantageous, and it might be related to the catabolism of mannitol and its use as a source of carbon. To elucidate whether this phenotype was specific for *Z. rouxii*, we compared the growth of *Sa. cerevisiae* BY4741 and its *stl1Δ* derivative on media supplemented with mannitol. As was observed with *Z. rouxii*, the growth of *Sa. cerevisiae* with the *stl1Δ* mutation on plates with 2% mannitol and 10 mM of glycerol was better than the growth of the corresponding wild type (data not shown).

Zr*Stl1p and* Zr*Stl2p are plasma-membrane glycerol transporters*

The heterologous expression of both genes in *Sa. cerevisiae* indicated that only *ZrSTL1* was a functional glycerol transporter. On the other hand, the characterisation of *Z. rouxii* mutants suggested that *Zr*Stl2p was involved in glycerol transport and/or metabolism as well. To solve this discrepancy, we constructed a series of plasmids for the expression of *ZrSTL* genes (behind their own promoters) in the *Z. rouxii* MS12 mutant and for tagging their products with GFP (Table S1). The growth of *Z. rouxii* MS12 mutants transformed with either an empty centromeric vector (pZCA) or with plasmids carrying the *ZrSTL1* and *ZrSTL2* genes was compared with the growth of UL4 harbouring the empty pZCA. Figure 4A shows that the presence of either *Zr*Stl1p or *Zr*Stl2p in MS12 cells complemented the phenotype of a higher sensitivity to NaCl. This complemented phenotype was much stronger when 10 mM of glycerol was added to the growth medium. Similar results were obtained with plasmids, where the GFP coding sequence was attached to the 3′ ends of Stl-coding sequences (not shown). The GFP tagging enabled the visualisation of both Stl proteins. As shown in Fig. 4B, both Stl1p and Stl2p were localised to the periphery of the *Z. rouxii* cells, confirming the role of both proteins as plasma-membrane transporters. The signal was observed in cells growing in YNB supplemented with glucose. This suggested again, that in *Z. rouxii* cells, the two *STL* genes and their products are neither repressed nor inactivated by glucose.

As we saw that the double MS12 mutant lost the ability to accumulate glycerol (Fig. 2C), we tested whether the reintroduction of deleted genes on plasmids would restore the ability to accumulate glycerol. Figure 4C shows that the reintroduction of both *STL* genes in centromeric plasmids restored the ability of the *Z. rouxii* MS12 strain (*stl1Δ stl2Δ*) to accumulate ^{14}C-glycerol. The accumulation of glycerol in MS12 cells expressing Stl2p was almost the same as in the UL4 (*STL1 STL2*) strain, and the presence of Stl1p resulted in a lower accumulation, but still significantly higher than in MS12 cells transformed with an empty vector (Fig. 4C).

Stl transporters contribute to Z. rouxii *intracellular pH homeostasis*

Intracellular pH is one of the most controlled parameters of cell physiology, controlling almost all processes occur-

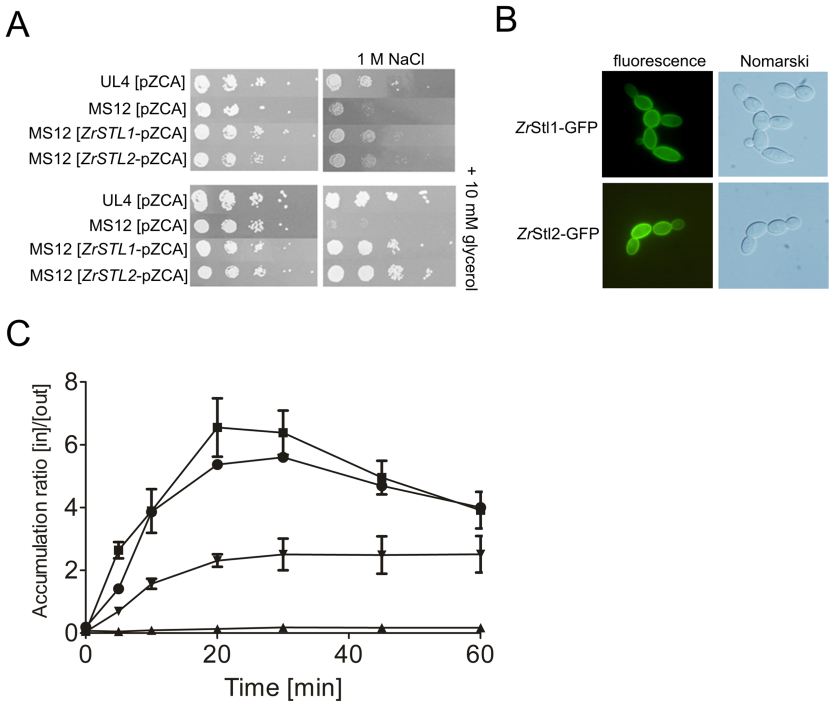

Fig. 4. Complementation of *stl* deletions.
A. Growth phenotypes of *Zygosaccharomyces rouxii* UL4 and MS12 transformed with centromeric empty vectors (pZCA) or vectors carrying *ZrSTL* genes (*ZrSTL1*-pZCA, *ZrSTL2*-pZCA) on YNB media with 2% mannitol and salt or glycerol added as indicated.
B. Localisation of *Zr*Stl1-GFP and *Zr*Stl2-GFP proteins expressed in *Z. rouxii* MS12 (*stl1Δ stl2Δ*) exponentially growing in minimal YNB supplemented with 2% glucose.
C. Accumulation of ^{14}C-glycerol in UL4 [pZCA] (●), MS12 [pZCA] (▲), MS12 [*ZrSTL1*-pZCA] (▼), and MS12 [*ZrSTL2*-pZCA] (■) growing in YNB supplemented with 2% glucose. Each point represents the mean ± SD of three biological replicates.

ring in cells, including the uptake of many different nutrients. Glycerol was originally suggested to be taken up in symport with Na^+ in *Z. rouxii* cells (van Zyl *et al*., 1990; Lages *et al*., 1999). However, we observed clear phenotypes of the deletion of *STL* genes in conditions where the concentration of sodium cations was negligible (e.g. media with high concentrations of sorbitol or mannitol). Thus, we assumed that the uptake of glycerol via Stl proteins might be driven by the proton gradient across the plasma membrane; i.e. the transport mechanism being a symport of glycerol with protons, similarly as in *Sa. cerevisiae* and *C. albicans*. To test this possibility, we first expressed pHluorin, a pH-sensitive variant of GFP (Miesenbock *et al*., 1998) in *Z. rouxii* cells and estimated the intracellular pH under various conditions (Fig. 5). First, we measured the intracellular pH in cells from the mid-exponential phase of growth in YNB-pH with 2% glucose, and we found that the pH of all three mutants lacking one or both *STL* genes was significantly higher than the pH of control UL4 cells (Fig. 5A). This result suggested that the higher intracellular pH was due to a diminished influx of protons via Stl transporters. The glycerol/H^+ symport meachanism was confirmed by monitoring the changes in extracellular pH of an unbuffered cell suspension upon the addition of a glycerol pulse (Fig. 5D). A fast alkalinisation of the external medium, i.e. the H^+ influx associated with glycerol uptake, was only observed when the UL4 (*STL1 STL2*) cells were used.

Then the effect of changing the growth medium on intracellular pH was monitored. Cells from the mid-exponential phase of growth in YNB-pH with 2% glucose were transferred to fresh YNB-pH medium supplemented with either 2% mannitol (Fig. 5B) or 2% glucose (Fig. 5C). When cells were transferred to YNB-pH medium supplemented with 2% mannitol as a source of carbon (Fig. 5B), the intracellular pH slightly dropped in all strains. In addition, on mannitol, as in the drop tests, the effect of *STL1* deletion on the intracellular pH was more significant than that of *STL2* deletion. This result again confirmed the prevailing role of Stl1p when cells grew on mannitol as a source of carbon. On the other hand, when the cells were transferred to the same fresh medium with 2% glucose, the intracellular pH rose in all strains (Fig. 5C). The difference between the wild type and mutants also increased (from approx. 0.1 to 0.2 pH units, cf. Fig. 5A and C). The increase in intracellular pH observed upon transferring the cells to fresh YNB medium supplemented with 2% glucose (Fig. 5C) suggested that *Z. rouxii* cells responded to the fresh media (and certain carbon sources) by increasing the activity of Pma1 H^+-ATPase, similarly to *Sa. cerevisiae* (Orij *et al*., 2009). Proton efflux through the *Sc*Pma1 ATPase is only activated by sugars metabolised in glycolysis (glucose, fructose and mannose) and not by e.g. galactose (Serrano, 1983). Figure 6A shows that *Z. rouxii* Pma1p behaved similarly to *Sa. cerevisiae* Pma1p and was only activated by fresh glucose and fructose. Neither *Z. rouxii*

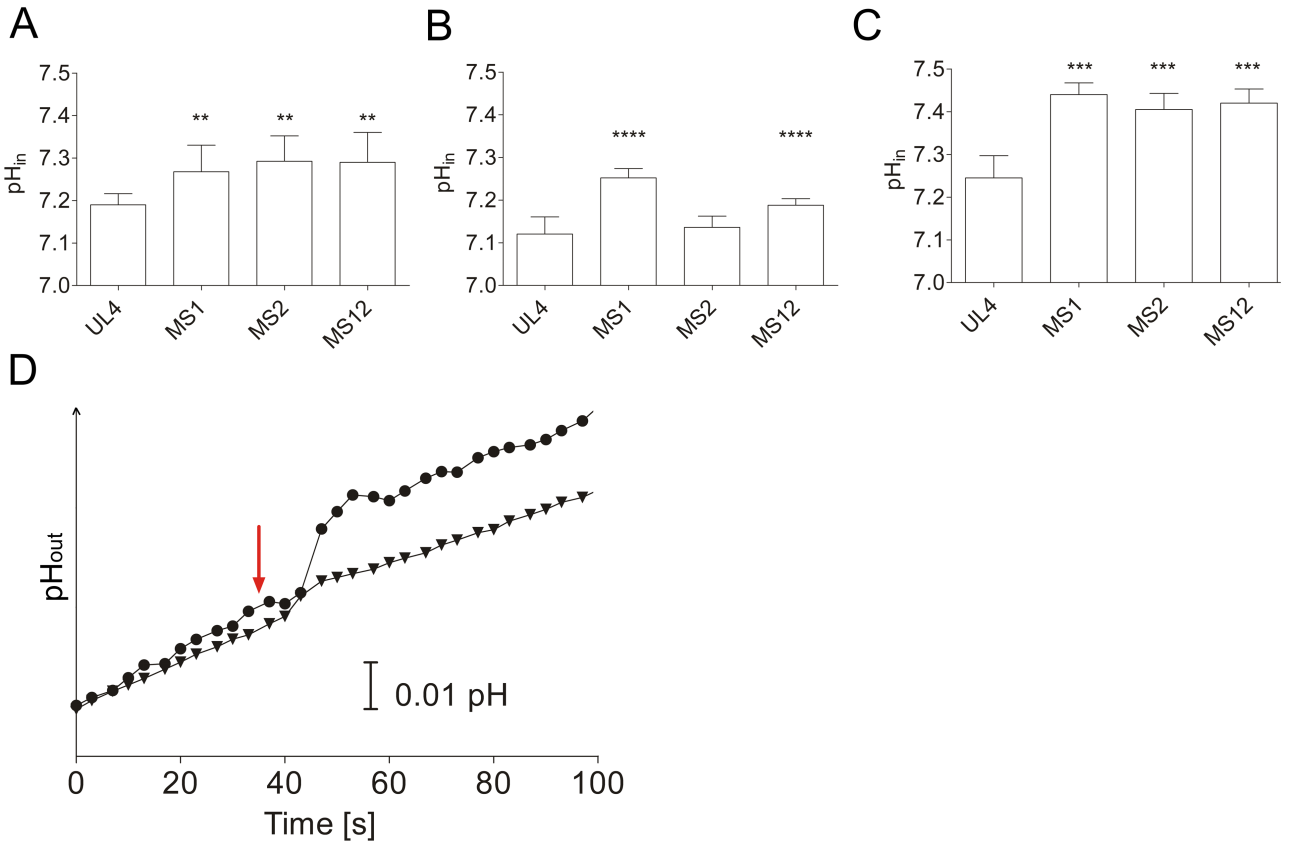

Fig. 5. Changes in intracellular and extracellular pH upon deletion of *STL* genes in *Zygosaccharomyces rouxii* cells. Intracellular pH of *Z. rouxii* UL4 and its *stl* derivatives (MS1, *stl1Δ*; MS2, *stl2Δ*; MS12, *stl1Δ stl2Δ*) was measured using the expression of pHluorin from the pZpH plasmid. The results are presented as the means ± SD of at least five biological replicates assayed in duplicate. **, $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ (two-tailed Student's *t*-test versus *Z. rouxii* UL4).
A. Intracellular pH of cells grown in YNB-pH with 2% glucose to $A_{600} = 0.5$.
B. Intracellular pH of cells pre-grown in YNB-pH with 2% glucose to $A_{600} = 0.5$ and then transferred into YNB-pH with 2% mannitol. pH was estimated after 2 h of incubation at 30°C.
C. Intracellular pH of cells pre-grown in YNB-pH with 2% glucose to $A_{600} = 0.5$ and then transferred into fresh YNB-pH with 2% glucose. pH was estimated after 2 h of incubation at 30°C.
D. Changes in external pH upon addition of glycerol. UL4 (●) and MS12 (*stl1Δ stl2Δ*, ▼) cells were suspended in water (pH adjusted to 5.3). The external pH was monitored with a pH electrode. Glycerol addition (final concentration 25.9 mM) is indicated by an arrow. Representative data of two biological replicates assayed in triplicate are shown.

nor *Sa. cerevisiae* cells showed any signs of Pma1p activation (i.e. a higher efflux of protons) by mannitol and galactose (Fig. 6A). This observation was confirmed by monitoring the acidification of external media after addition of various carbon sources (Fig. 6B). As shown in Fig. 6B, only the addition of fructose and glucose resulted in a significant increase in the acidification of external media.

Taken together, our data obtained in pH measurements indicated (i) that glycerol enters *Z. rouxii* cells via Stl proteins in symport with protons, (ii) that the deletion of *STL* genes influenced the intracellular pH and thus probably the overall metabolism and growth of *Z. rouxii* even under standard non-stressed conditions, and (iii) that *Zr*Pma1p is activated by glucose and fructose similarly as *Sc*Pma1p.

Expression of ZrSTL1 *is highly regulated whereas* ZrSTL2 *is more constitutive*

To elucidate the regulation of the expression of *Zr*Stl1 and *Zr*Stl2 glycerol transporters, we analysed the level of expression of the corresponding genes in *Z. rouxii* UL4 cells grown under various conditions. In the first experiment, we tested the expression of both genes in cells grown up to the mid-exponential phase in YP supplemented with various sources of carbon or by a moderate (0.4 M) concentration of NaCl. The results obtained showed that both genes, and especially *ZrSTL1*, were upregulated when a carbon source such as mannitol was used (Fig. 7A). Surprisingly, a mild long-term hyperosmotic stress caused by 0.4 M NaCl resulted in a much lower increase in expression.

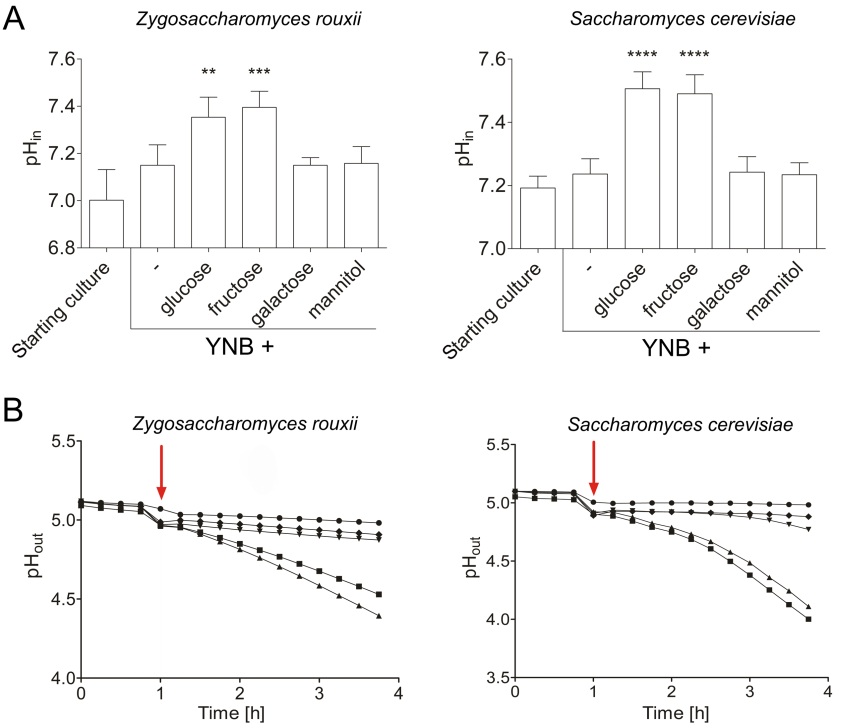

Fig. 6. Activation of Pma1 in *Zygosaccharomyces rouxii* and *Saccharomyces cerevisiae* cells.
A. Changes in intracellular pH of *Z. rouxii* UL4 [pZpH] and *Sa. cerevisiae* AQ [pVT-pH] after transfer to the fresh YNB medium with indicated 2% carbon source. Starting culture, cells grown in YNB + 2% glucose to $A_{600} = 0.5$ for *Z. rouxii*, and to $A_{600} = 0.4$ for *Sa. cerevisiae*. The results are presented as the means ± SD of at least two biological replicates assayed in triplicate. **, $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ (two-tailed Student's *t*-test versus starting culture).
B. Acidification of YNB medium by *Z. rouxii* UL4 and *Sa. cerevisiae* AQ after addition of 2% carbon sources (■, glucose; ▲, fructose; ▼, galactose; ◆, mannitol) or water (●, negative control). The addition of a carbon source is indicated by an arrow. Representative data of two biological replicates assayed in triplicate are shown.

To monitor changes in expression, YPD pre-grown cells were transferred into fresh YP media with the indicated carbon sources and the level of *STL* transcripts was estimated after 30 min of incubation. As shown in Fig. 7B, mannitol, sorbitol, glycerol and maltose elicited a rapid increase in the expression of both genes, but again more significantly for *ZrSTL1*. Unlike the first experiment, both genes were substantially upregulated within 30 min after the transfer of cells to a medium with 1 M salts (Fig. 7B). Figure 7C shows a very important difference in the expression of *Z. rouxii STL* genes. Only *ZrSTL1* seemed to be repressed by fructose, whereas the expression of *ZrSTL2* is similar in the presence of fructose and glucose.

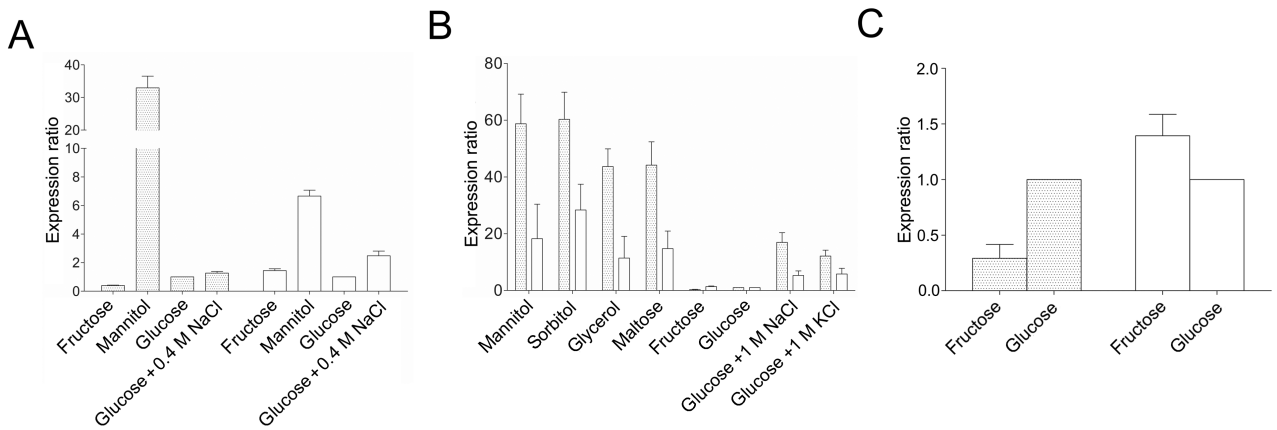

Fig. 7. Expression of *ZrSTL1* (shaded bars) and *ZrSTL2* (open bars) was followed by qRT-PCR in UL4 cells. The mRNA levels were normalised to *ZrACT1*. The expression ratio is presented as the multiple of the expression in the presence of 2% glucose. The results are presented as the mean ± SD of three biological replicates assayed in triplicate.
A. Expression levels in cells from mid-exponential growth phase in YP media with 2% indicated carbon sources.
B. Expression levels in cells pre-grown in YPD ($A_{600} = 0.6$) and subsequently transferred to YP media supplemented with 2% carbon sources as indicated or to YPD + 1 M NaCl/KCl for 30 min.
C. Relative expression of *ZrSTL1* and *ZrSTL2* genes in cells from exponential phase of growth in YP media with 2% glucose or 2% fructose.

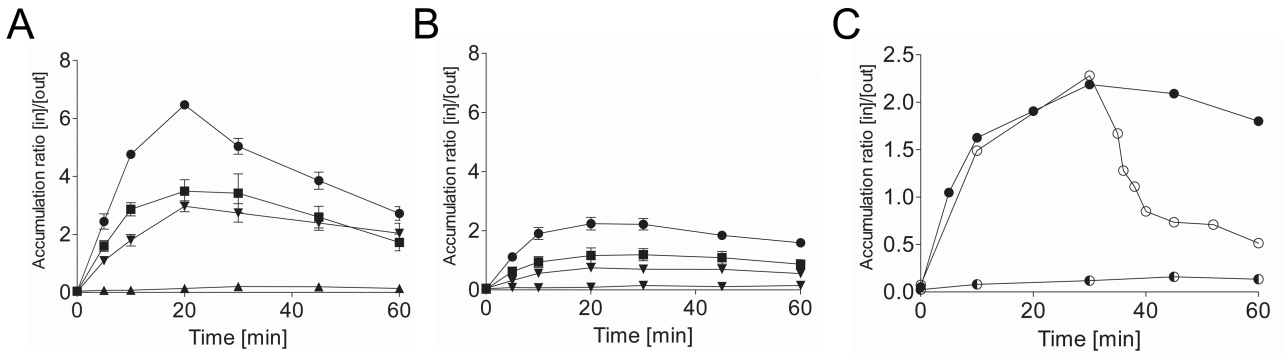

Fig. 8. Accumulation of ^{14}C-glycerol in UL4 (●), MS1 (*stl1Δ*, ■), MS2 (*stl2Δ*,▼), and MS12 (*stl1Δ stl2Δ*,▲) cells. Each point represents a mean ± SD of three biological replicates.
A. Accumulation of ^{14}C-glycerol in cells from the exponential growth phase in YP + 2% glucose.
B. Accumulation of ^{14}C-glycerol in cells from the exponential growth phase in YP + 2% fructose.
C. Accumulation of ^{14}C-glycerol in UL4 cells growing in YEPG. The accumulation was monitored in the absence (close symbols) or in the presence (semi-opened symbol) of 50 μM CCCP. Open symbols represent the changes in accumulation ratios upon addition of 50 μM CCCP, 35 min from the beginning of experiment.

To confirm the results obtained in RT-PCR experiments, we measured the accumulation ratios of glycerol in cells grown in YP with either glucose or fructose as a source of carbon. As shown in Fig. 8, glycerol was accumulated in higher levels in cells grown in glucose than in cells grown in fructose. The results obtained were in agreement with the observed repression of the *ZrSTL1* gene in the presence of fructose (Fig. 7C). To reconfirm the active mechanism of glycerol uptake and the proton motive force as a source of energy, the accumulation of glycerol in UL4 cells was monitored in the presence of carbonyl cyanide m-chlorophenylhydrazone (CCCP), a protonophore that was added either together with radiolabelled glycerol or 35 min later. As shown in Fig. 8C, glycerol accumulation was completely prevented in the presence of CCCP, and the already accumulated glycerol was released when the inward gradient of protons was dissipated.

A detailed kinetic characterisation of ^{14}C-glycerol uptake via *Zr*Stl1p and *Zr*Stl2p revealed that both transporters have similar affinity to glycerol in the mM range (Table 2), although *Zr*Stl1p seemed to have a slightly higher affinity and lower capacity (Table 2, Fig. S3A). The measurement of initial uptake rates in *Z. rouxii* MS2 (*stl2Δ*) showed that apart from Stl1p, there was present a third, low-affinity transporter (Table 2, Fig. S3B).

Fructose produced by Z. rouxii *invertase blocks glycerol accumulation*

To further confirm the results obtained in RT-PCR experiments, we monitored the uptake of glycerol in cells grown with sucrose as a source of carbon. Yeasts usually do not contain a sucrose transporter. Instead, *Sa. cerevisiae* and some other yeast species produce the extracellular enzyme invertase to break sucrose down into two monosacharides, glucose and fructose (Albertyn *et al*., 1994) which are taken up by specific hexose transporters. We found a homologue of the invertase-encoding *ScSUC2* gene (YIL162w) in the genome of *Z. rouxii* (ZYRO0B13376g). Based on this finding and on the fact that *Z. rouxii* grows with sucrose as a source of carbon, we expected the production of invertase by *Z. rouxii*. With glucophilic *Sa. cerevisiae*, glucose is preferentially transported and metabolised, whereas in fructophilic *Z. rouxii*, fructose is used prior to glucose (Emmerich and Radler, 1983; Leandro *et al*., 2014). We measured the accumulation of glycerol during the growth of *Z. rouxii* UL4 in YP + 50% sucrose, i.e. in the presence of hyperosmotic stress. We found that the glycerol accumulation differed depending on the growth phase (Fig. S4). At the beginning of growth (growth phase I), cells produced invertase and

Table 2. Kinetic parameters of glycerol uptake in cells from the exponential phase of growth in YPD (A_{600} = 0.6).

Strain	Transporter	K_m (mM)	V_{max} (μmol.h^{-1}g d.w^{-})
Zygosaccharomyces rouxii MS1	Stl2p	3.99 ± 0.52	145.00 ± 11.75
Zygosaccharomyces rouxii MS2	Stl1p	0.73 ± 0.30	46.21 ± 8.38
	Unknown transporter	28.60 ± 6.51	546.10 ± 92.99

sucrose was broken down into glucose and fructose. The glycerol uptake was negligible at this point because of the high concentration of fructose, which is taken up by cells before glucose (Leandro *et al*., 2014). This result also showed that the repression (and/or inactivation) of *Zr*Stl proteins by fructose is stronger than the upregulation of their experession caused by the high osmotic pressure (50% sugars in the medium). Over time, fructose was successively consumed and cells then started to transport and metabolise glucose (growth phase II; Leandro *et al*., 2014) and accumulate radiolabelled glycerol. When both monosaccharides were consumed (growth phase III), cells continued to transport glycerol. These results supported the finding that the uptake of glycerol is affected more by fructose than by glucose in fructophilic *Z. rouxii*.

Discussion

Active glycerol uptake systems are believed to exist in most yeast species. The name of the transporters, Stl, is the abbreviated version of Sugar Transporter Like and is based on their similarity to hexose transporters (Zhao *et al*., 1994). Members of the Stl family share a strong structural conservation typical of hexose transporters such as 12 transmembrane domains (TMD) and five sequence-conserved motifs (Zhao *et al*., 1994; Leandro *et al*., 2009). A detailed search for putative glycerol Stl transporters in the sequenced genomes of various yeast species revealed that the genomes of osmotolerant yeasts usually contain more than one copy of a homologous gene [e.g. eight in *D. hansenii*, six in *Yarrowia lipolytica* or three in *Pichia stipitis* (Fig. 9, Table S3 and Palma *et al*., 2007)]. Subsequent analysis showed that identified Stl proteins can be divided into three subfamilies (Fig. 9). The first and biggest subfamily, named '*Sc*Stl1' based on the high level of identity with *Sc*Stl1p, contains transporters sharing approximately 60% identity, including *Ca*Stl1p as well as both putative Stl transporters from *Z. rouxii*. The second and more distant subfamily ('*Ca*Stl2') involves proteins highly similar to *Ca*Stl2p. The level of identity among the members of this subfamily is between 50% and 60%. Neither the transport function nor the physiology role of any of them has been fully characterised. Recently described proteins from *P. tannophilus* are also members of this subfamily, but their function in glycerol uptake or homeostasis has not been confirmed (Liu *et al*., 2013). The third subfamily includes proteins with a relatively low level of identity to the first two groups (around 27%) and a completely unknown role in cells.

The position of the two *Z. rouxii* homologues within the first subfamily, which contains the only two transporters to have so far been characterised from *S. cereveisiae* and *C. albicans*, suggested their function in active glycerol uptake. Our results provide several lines of evidence supporting the conclusion that *Z. rouxii* takes up glycerol actively via two H^+ symporters, the proteins Stl1 and Stl2.

Our first approach, the expression of the *Z. rouxii STL* genes in *Sa. cerevisiae* cells, was only partly successful. Introduction of the *ZrSTL1* gene into *Sa. cerevisiae* mutants resulted in clear phenotypes corresponding to the presence of an active glycerol uptake system (Fig. 1A and C), and measurements of glycerol accumulation confirmed this conclusion (Fig. 1B and D). On the other hand, *STL2* expression neither complemented the salt-sensitivity nor the absence of active glycerol accumulation (Fig. 1). As for the 87 amino acid longer hydrophilic N-terminus of Stl2p (Fig. S1), we first hypothesised that Stl2p may serve as a sensor of external glycerol, like the *Sa. cerevisiae* sensors for amino acids [Ssy1p; (Klasson *et al*., 1999)] or sugars [Rgt2p, Snf3p; (Ozcan *et al*., 1996)] that are only distinguished from relevant groups of transporters by longer hydrophilic N- or C- termini. Nevertheless, experiments with *Z. rouxii* cells showed that *Zr*Stl2p is an active glycerol transporter like the *Zr*Stl1 and not a glycerol sensor.

The construction of *Z. rouxii* mutants lacking one or both of the *STL* genes revealed that the absence of *STL1* and *STL2* strongly affected the growth of mutants under osmotic stress (e.g. Fig. 4, 1 M NaCl, Fig. S 2B) and their ability to actively accumulate glycerol (Figs. 2C, 4C). The observed osmosensitivity of the *Z. rouxii* mutants lacking *STL* genes seems to be rather species-specific; the growth of the *C. albicans stl1Δ* homozygous mutant in mimimal YNB medium supplemented with 1 M NaCl is not inhibited (Kayingo *et al*., 2009). The *Z. rouxii* wild-type phenotype of salt tolerance and the ability to accumulate glycerol were restored in the MS12 cells (*stl1Δ stl2Δ*) by the reintroduction of the *STL1* or *STL2* genes (Fig. 4A and C). These results showed that Stl1 and Stl2 are both glycerol transporters. Moreover, our comparison of the wild type and single and double mutants indicated that the *Z. rouxii* Stl proteins are active glycerol transporters whose physiological role is not limited to the accumulation of a compatible solute during hyperosmotic stress, but whose activity contributes to cell fitness even under standard, non-stressed growth conditions (Figs. 2A, 2B, 5A, Fig. S2B). *Z. rouxii* cells needed functional Stl transporters for their growth on minimal media, and this need was much stronger in cells with multiple auxotrophies (DLA2, Fig. S2B). In *Z. rouxii*, observed phenotypes might be related to the cell metabolism in auxotrophes and probably connected, like in *Sa. cerevisiae*, with an altered redox homeostasis (Vandijken and Scheffers, 1986; Ansell *et al*., 1997; Larsson *et al*., 1998). It is worth noting that, unlike *Sa. cerevisiae*, *Z. rouxii* CBS 732^T cells do not use glycerol as a source of carbon (Kurtzman *et al*., 2010; Bubnova *et al*., 2014), and thus the role of *Zr*Stl transporters is not nutritive. Both *C. albicans* and *Sa. cerevisiae* do not seem to be affected by the absence of the *STL1* gene under non-

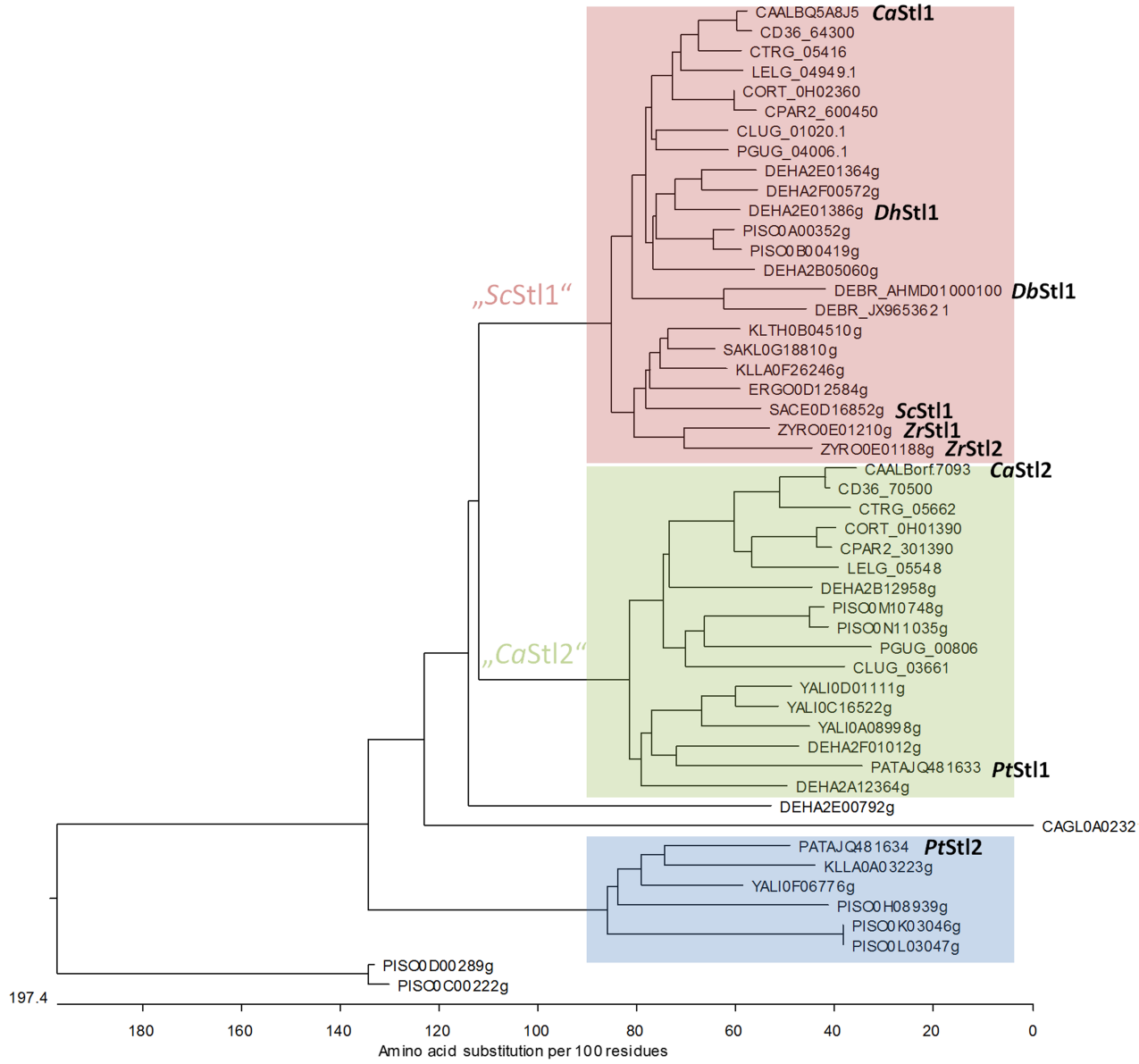

Fig. 9. Phylogenetic tree of Stl proteins from *Zygosaccharomyces rouxii* CBS 732^T (ZYRO), *Saccharomyces cerevisiae* S288C (SACE), *Candida albicans* SC5314 (CAALB), *Debaryomyces hansenii* CBS 767 (DEHA), *Pachysolen tannophilus* CBS 4044 (PATA) and uncharacterised homologues of Stl1 proteins from *Candida glabrata* CBS 138 (CAGL), *Candida dubliniensis* CD36 (CD36), *Candida tropicalis* MYA-3404 (CTRG), *Candida parapsilosis* CDC317 (CPAR), *Candida orthopsilosis* Co 90–125 (CORT), *Candida guilliermondii* ATCC 6260 (PGUG), *Candida lusitaniae* ATCC 42720 (CLUG), *Dekkera bruxellensis* CBS 2499 (DEBR), *Pichia sorbitophila* CBS 7064 (PISO), *Yarrowia lipolytica* CLIB122 (YALI), *Kluyveromyces lactis* CLIB210 (KLLA), *Kluyveromyces thermotolerans* CBS 6340 (KLTH), *Saccharomyces kluyveri* CBS 3082 (SAKL) and *Lodderomyces elongisporus* NRLL YB-4239 (LELG). SeqBuilder and MegAlign were used to see, edit and align sequences (Clustal W method).

stressed conditions (Ferreira and Lucas, 2007; Kayingo *et al*., 2009; Duskova *et al*., 2015). In *Z. rouxii*, the strongest osmosensitivity of *stl* mutants was observed when mannitol was used as a source of carbon (e.g. Figs. 3C and 4A). The presence of at least one Stl glycerol uptake system and a low amount of glycerol (10 mM) was necessary to support the growth of cells in media with high concentrations of salts or other solutes (Fig. 3C). On the other hand, the absence of the *STL1* gene (strains MS1 and MS12) was advantageous for cell growth in minimal medium supplemented with only 2% mannitol and 10 mM glycerol; i.e. without an osmotic stress (Fig. 3C; bottom left panel). This surprising phenotype is probably due to the catabolism of mannitol and glycerol. It was shown that

glycerol formation may occur in *Z. bailii* under strictly aerobic conditions (Vandijken and Scheffers, 1986) and the situation may be the same in its close relative *Z. rouxii*. When mannitol is catabolised by mannitol dehydrogenase, NAD(P)H is generated, and to restore the $NAD(P)^+$/NAD(P)H equilibrium, glycerol is usually generated in yeast cells (Vandijken and Scheffers, 1986; Quain and Boulton, 1987; Perfect *et al*., 1996). Under the conditions used in our experiments with *Z. rouxii* cells, mannitol as a source of carbon was catabolised and thus surplus NAD(P)H was probably generated. As external glycerol was simultaneously taken up via Stl transporters (and mainly via Stl1p, cf. Fig. 7, expression on mannitol), it was probably synthesised less to restore the $NAD(P)^+$. Another reason for a slower growth of the wild type on the medium with mannitol and glycerol may be the competition of both compounds for the energy source necessary for the active uptake (proton motive force). As nothing is known on the mechanism of mannitol transport and metabolism, or on the maintenance of redox balance in *Z. rouxii* cells, our presumption is only based on the known pathways in *Sa. cerevisiae*, and mannitol transport and its catabolic pathways together with the metabolic basis for glycerol formation or degradation in *Z. rouxii* should be elucidated.

Both *Z. rouxii* Stl transporters had similar kinetic parameters (Table 2), were localised to the plasma membrane (Fig. 4B), contributed similarly to glycerol uptake, osmotolerance (Figs. 2C, 3 and 4A), cell fitness and survival of hypoosmotic shock (Fig. 2D), and surprisingly, the presence of both was necessary to maintain intracellular pH (Fig. 5A and C). Nevertheless, Stl1p seemed to have a predominant role when non-fermentable sources of carbon were used (Fig. 7B). Its expression was highly upregulated (Fig. 7A and B) and its activity predominated when cells were grown e.g. with mannitol (Figs. 3C and 5B). The *stl1Δ* mutant also produced more glycerol than *stl2Δ* cells under standard growth conditions (Fig. 2B; YPD medium). Expression of the *STL1* gene changed more with varying growth conditions (Fig. 7A and B) and was repressed more efficiently by the presence of fructose (Fig. 7C).

Almost a quarter of a century ago, glycerol was believed to be transported through the cytoplasmic membrane in symport with Na^+ in *Z. rouxii* cells (van Zyl *et al*., 1990). Also for several other salt-tolerant yeast species, e.g. *D. hansenii* (Lucas *et al*., 1990), glycerol was presumed to be actively taken up together with sodium cations (Lages *et al*., 1999). In all of those experiments, the uptake of glycerol was measured in the presence of NaCl. Here we show that both *Z. rouxii* Stl transporters use the inward gradient of protons across the plasma membrane created by the Pma1 H^+-ATPase (Fig. 5D). The phenotypes of Stl activity were observed in the absence of salts (e.g. Fig. 3 and S4), the accumulation of glycerol was inhibited by the presence of a protonophore (Fig. 8C), and the absence of Stl transporters affected the intracellular pH (Fig. 5A–C) and the alkalinisation of external medium upon glycerol addition (Fig. 5D).

Taken together, our results showed that the osmotolerant and fructophilic yeast *Z. rouxii* employs two plasma-membrane transport systems, Stl1p and Stl2p, mediating the active uptake of glycerol with protons. These transporters are highly important, though *Z. rouxii* CBS 732^T cells do not use external glycerol as a source of carbon (Kurtzman *et al*., 2010; Bubnova *et al*., 2014). Both transporters participate not only in the high osmotolerance of this species, as expected, but their activity also contributes to the survival of a hypoosmotic stress. Unlike in *Sa. cerevisiae*, the two Stl transporters are also active in *Z. rouxii* cells grown under standard non-stressed conditions (YPD or YNB with glucose) and their activity contributes to the maintenance of glycerol and intracellular pH homeostases. The expression of Stl1p is tightly regulated, being highly activated in non-fermentable carbon sources and downregulated by fructose. The expression of Stl2p is less regulated and resembles those of house-keeping genes. The characterisation of active glycerol uptake and the properties of the transporters involved in osmotolerant *Z. rouxii* may serve as the basis for improving the osmotolerance of the industrial yeast strains used in fermentation processes in environments with a low water activity.

Experimental procedures

Yeast strains and growth media

The yeast strains used in this study are listed in Table 1. Mutant strains of *Z. rouxii* lacking *STL1*, *STL2* or both are derivatives of *Z. rouxii* UL4 (*ura3*) and *Z. rouxii* DLA2 [*ura3 leu2Δ::loxP ade2Δ::loxP*; (Pribylova and Sychrova, 2003; Pribylova *et al*., 2007b)]. The *ZrSTL1* and *ZrSTL2* genes were deleted by homologous recombination using the Cre-*loxP* system (Guldener *et al*., 1996) with the *KanMX* marker gene, then pZCRE (Pribylova *et al*., 2007b) and the primers listed in Table S2. *Escherichia coli* XL1-Blue (Stratagene, Agilent Technologies, Santa Clara, CA, USA) was routinely used as the host for plasmid amplification.

Yeast batch cultures were grown aerobically (30°C, 160 r.p.m.) in complex YPD (1% yeast extract, 2% peptone, 2% glucose), YEPG (1% yeast extract, 2% peptone, 1% ethanol, 1% glycerol) or minimal YNB (0.67% YNB without amino acids, 2% glucose). For growth test on various carbon sources, fructose, mannitol, sorbitol, maltose and galactose at the indicated concentration were in the media instead of glucose. The addition of mannitol, sorbitol, NaCl or KCl (at concentrations indicated in the text) was used to test growth under high osmotic pressure. YNB-pH (0.17% YNB without ammonium sulphate, without folic acid, without riboflavin (MP Biomedicals); 0.4% ammonium sulphate) supplemented with indicated 2% source of carbon was used to prepare cultures for the measurement of intracellular pH. When required, auxotrophic supplements BSM (Brent Supplement Mix, Formedium™, Hunstanton, UK), BSM without uracil or G418

(100 μg ml^{-1}, Formedium™, UK) were used for transformant selection. Solid media contained 2% agar. *E. coli* transformans were grown in standard Luria-Bertani medium supplemented with ampicillin (100 μg ml^{-1}).

Plasmids

The plasmids used in this study are listed in Table S1. All new plasmids were constructed by homologous recombination in *Sa. cerevisiae* BW31a. The primers for the construction of all plasmids are listed in Table S2. Genomic DNA of *Z. rouxii* UL4 was isolated as described earlier (Hoffman and Winston, 1987) and used as a template for the amplification of the required fragments containing *ZrSTL1* or *ZrSTL2* genes. *ZrSTL1* and *ZrSTL2* with their own promoters (2000 nt long) were inserted into two *Z. rouxii* vectors (pZCA, pZGFP). All constructed plasmids were verified by PCR using diagnostic primers and by sequencing.

Growth assays

The growth of yeast cells was monitored on solid and in liquid media. The growth rate measurements in liquid media were performed in 96-well plates according to (Maresova and Sychrova, 2007). For drop tests on solid media, cells pregrown on YPD or on YNB plates at 30 °C for 48 h were suspended in sterile water to A_{600} = 2 (Spekol 211, Carl Zeiss, Jena, Germany). Serial 15-fold dilutions of cell suspensions were spotted on YPD or YNB plates without or supplemented with various carbon sources, salts or glycerol as indicated. All experiments were repeated at least three times and representative results are shown.

Survival of stress

To determine the tolerance of *Z. rouxii* wild-type and mutant strains to hyperosmotic and hypoosmotic stresses, cells were grown to A_{600} = 0.6 in 50 ml of YPD. Cultures were transferred to YP + 1 M of mannitol or YPD + 1 M of KCl for 16 h and then to H_2O for 45 min. In each step of this procedure, the A_{600} of cells was adjusted to 1.0 and subsequently, 50 μl of 2000-fold and 100 μl of 20,000-fold diluted aliquots were plated on YPD in triplicate. The number of colonies (CFU) was counted after 2 days of growth. The experiment was repeated three times and the means ± SD are shown.

Glycerol production

To analyse the total production of glycerol, cells were grown in three parallels in 20 ml of YPD media to A_{600} = 1. Two aliquots of 1 ml were then withdrawn, boiled for 10 min, mixed intensively with a vortex and centrifuged (14,000 *g*; 10 min). The glycerol concentration was measured in the supernatant using an EBO glycerol kit (Roche CZ, Prague, Czech Republic). The presented results are the means ± SD of three biological replicates assayed in triplicate.

Glycerol uptake and accumulation

To estimate the initial rates of uptake of radiolabelled glycerol and the [in]/[out] accumulation ratios, we used the methods described earlier (Lages and Lucas, 1995). Cells grown in 100 ml of media (indicated in the text) were harvested in the mid-exponential growth phase (usually A_{600} = 0.6; A_{600} = 0.4 for *Sa. cerevisiae* grown in YNB without uracil), washed twice with ice-cold distilled water and resuspended to a final concentration of about 30 mg (dry weight) ml^{-1} in distilled water. To follow the initial uptake rates or glycerol accumulation, aqueous solutions of ^{14}C-glycerol ([^{14}C(U)] glycerol, ARC, St Louis, MO, USA) with specific activities of 200 dpm nmol^{-1} were used. The initial uptake rates were estimated in triplicate for each glycerol concentration, and related to a blank value obtained by inverting the sequence of glycerol and cold water addition to the cell suspension. Reported data are the mean ± SD of at least five biological replicates. Kinetic parameters were estimated from Eadie–Hofstee plots and confirmed by computer nonlinear regression analysis using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). The ability to release accumulated radiolabelled glycerol was assayed with 50 μM protonophore CCCP added either 35 min after, or simultaneously with the radiolabelled glycerol. The experiments estimating the glycerol accumulation ratios were repeated at least three times and the means ± SD are presented. The radioactivity of cell samples was measured in vials containing 5 ml of scintillation counting cocktail (Opti-Phase HiSafe II, LKB FSA Laboratory Supplies, Loughborough, UK) in a Packard Tti-Carb 2200CA liquid scintillation spectrophotometer (PerkinElmer, Waltham, MA, USA).

H^+ movements associated with glycerol uptake

The existence of an H^+ influx associated with initial glycerol uptake was monitored as described previously (Loureiro-Dias and Peinado, 1984; Leandro *et al.*, 2013). Cells grown in 100 ml of YPD were harvested in the mid-exponential growth phase (A_{600} = 0.7–0.8), washed twice with ice-cold distilled water, resuspended to a final concentration of about 25 mg (dry weight) ml^{-1} in ice-cold distilled water and kept on ice; 0.9 ml of cell suspension was mixed with 1.36 ml of water and pH was adjusted to 5.3 with HCl. Cells were incubated in a heated chamber with magnetic stirring (Oxygraph-2K; OROBOROS® Instruments, Innsbruck, Austria) at 25°C. After obtaining a baseline, 60 μl of 1 M glycerol was added (final concentration 25.9 mM), and pH data were collected with a pH electrode (Hamilton, Reno, NV, USA) connected to OROBOROS Oxygraph-2K in number of second intervals. The experiment was repeated twice in triplicate and representative results are shown.

Intracellular pH

The intracellular pH was determined in *Z. rouxii* and *Sa. cerevisiae* transformed with the pZpH, and pHl-U plasmids, respectively, carrying the sequence of pHluorin (Maresova *et al.*, 2010; Stribny *et al.*, 2012). The calibration curves for *Sa. cerevisiae* and *Z. rouxii* were generated as described previously with some modifications (Orij *et al.*, 2009). Ten millilitres of cells grown to A_{600} = 0.5 in YNB–pH medium with appropriate auxotrophic supplements were harvested, washed with 10 ml of PBS (Sigma CZ, Prague, Czech Republic)) and resuspended in 10 ml of PBS containing digitonin

(450 μg ml^{-1} for *Z. rouxii*; 150 μg ml^{-1} for *Sa. cerevisiae*). Digitonin was washed out after 17 min (*Z. rouxii*) or 8 min (*Sa. cerevisiae*); cells were resuspended in 1 ml of PBS and kept on ice. The wells of the ELISA plate (flat-bottom, PS) were filled with 10 μl of cell suspension and 90 μl of eight citric acid/Na_2HPO_4 buffers of different pH (ranging from 5.7 to 7.7; McIlvaine's buffer system). The fluorescence intensities were recorded using a Synergy HT microplate reader (BioTek Instruments, Winooski, VT, USA) with a 516/20 nm emission filter and 400/30 nm and 485/20 nm excitation filters. For eliminating the background fluorescence, wild-type cultures with an empty vector (pZEU for *Z. rouxii*, pVT100-U for *Sa. cerevisiae*) were grown and treated in parallel, and their fluorescence values were subtracted from the fluorescence at each excitation wavelength (software Gen 5, BioTek Instruments, Winooski, VT, USA). Calibration curves were obtained by plotting the ratio of emission at both excitation wavelengths ($I_{400/485}$) against the pH of buffers (data were fitted to a third-degree polynomial regression).

To estimate the pH of the cytosol, cells were grown in YNB-pH with 2% glucose to the mid-exponential growth phase (A_{600} = 0.5–0.6 for *Z. rouxii*, A_{600} = 0.4 for *Sa. cerevisiae*). The pH was estimated directly in the growing culture using the 96-well plates, and the background fluorescence was estimated and subtracted as described earlier. The results are presented as the means ± SD of at least five biological replicates assayed in duplicate.

Acidification of external medium

The acidification of media upon addition of various carbon sources was performed as described previously (Maresova and Sychrova, 2007). *Z. rouxii* UL4 and *Sa. cerevisiae* AQ cells from overnight cultures grown in YPD were washed twice with YNB without carbon source and resuspended to final A_{600} = 0.1 in the same medium containing pH indicator (0.01% bromcresol green sodium salt). After 60 min, the indicated carbon sources (5 μl of 40% solution of glucose, fructose, galactose and mannitol) or 5 μl od distilled water (negative control) were added. The changes in absorbance (A_{595}) were recorded in an ELx808 Absorbance Microplate Reader (BioTek Instruments). The experiment was done in two biological replicates assayed in triplicate. The calibration and external pH calculations were performed as in (Maresova and Sychrova, 2007).

Gene expression (qRT-PCR)

For qRT-PCR experiments, *Z. rouxii* UL4 cells were grown in different types of media up to the mid-exponential growth phase. Three 2 ml aliquots from each culture were centrifuged and the mRNA from the pellet immediately isolated using RNA Blue (Top-Bio, Prague, Czech Republic) following the manufacturer's instructions. The concentration and quality of obtained mRNA was verified by NanoDrop spectrophotometer (NanoDrop Products, Wilmington, DE, USA). The concentration of isolated mRNA was adjusted to the same concentration for all samples and the remaining DNA was removed by DNase I treatment (DNase I, without RNase, Top-Bio). The mRNA samples were reverse-transcribed to cDNA with a high-capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA). The cDNA samples were quantified by real-time PCR in a ViiA™ 7 Real-Time PCR System (Life Technologies) using 5× HOT FIREPol® Probe qPCR Mix Plus (ROX; Solis BioDyne, Tartu, Estonia) and TagMan Assays (Generi-Biotech, Hradec Kralove, Czech Republic) specific for *Z. rouxii* UL4's *ZrSTL1* (ZrSTL1_Q2, Order No: 00187-13), *ZrSTL2* (ZrSTL2_Q1, Order No: 00187-13) and *ZrACT1* (ZrACT1_Q1, Order No: 00187-13). The expression of the *ZrACT1* gene, encoding actin, was estimated in each experiment to normalise the mRNA expression in each sample. Each PCR reaction was performed in triplicate in a final volume of 10 μl using target genes labelled with FAM. The quantity of the transcript was determined using the standard curve method with 10-fold dilution of the mixed cDNA sample. The results are presented as the means ± SD of three biological replicates assayed in triplicate.

Fluorescence microscopy

Yeast cells producing Stl proteins tagged with C-terminal GFP were grown in YNB with 2% glucose to the exponential growth phase. The fluorescence signal was observed under an *Olympus* AX (Olympus Corporation, Tokyo, Japan) 70 microscope using a U-MWB cube with a 450–480 nm excitation filter and 515 nm barrier filter or under Nomarski contrast.

In silico *analyses*

The sequences of DNA and proteins were obtained from the Génolevures (Sherman *et al.*, 2009), SGD (*Saccharomyces* Genome Database; Cherry *et al.*, 2012) and CGD (*Candida* Genome Database Inglis *et al.*, 2012) databases. The multiple sequence alignment and comparative analyses were performed using the DNASTAR Lasergene 11 Core Suite (DNASTAR, Madison, WI, USA). The TMHMM (TransMembrane prediction using Hidden Markov Models) server v. 2.0 was used to predict the number of transmembrane domains (http://www.cbs.dtu.dk/services/TMHMM/).

Statistics

Data were analysed in GraphPad Prism 5 and *P*-values were calculated using the two-tailed Student's *t*-test.

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